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Full Length Research Paper

Development of humanized monoclonal antibody by logical approach: Characterization, functional studies *in vitro* and immunogenicity studies *in vivo* in non-human primates

Suba Dharshanan[1,2]*, Heilly Chong[1], Cheah Swee Hung[2] and Zulkeflie Zamrod[3]

[1]Protein Science Department, Inno Biologics, Nilai, Negeri Sembilan, Malaysia.
[2]Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur Malaysia.
[3]Inno Bio Ventures, Damansara, Kuala Lumpur, Malaysia.

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Monoclonal antibodies (mAbs) are widely used due to their exquisite specificity and once were hailed as the solution to cancer. However, when mouse mAbs are administered in humans, anti-antibody response (AAR) is frequently observed. Using humanized mAbs which are commonly developed by CDR-grafting method, the AAR is negligible, but loss of binding has also been consistently reported. Therefore, in an effort to produce humanized anti-C2 mAbs that retain binding properties but produce minimal AAR, humanized mAb has been developed by logical approach using IgBLAST. The purity and functionality of humanized mAb was confirmed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and cell-based assays, respectively. Although the humanized mAb developed using logical approach had reduced AAR compared to mouse anti-C2 mAb in *Macaca fascicularis*, however the AAR was higher, compared to humanized mAb developed using deimmunization method. Hence, for the development of functional humanized mAbs with negligible AAR, it is recommended that amphipathic mouse residues, excluding those located in the CDR or Vernier zone, be chosen for humanization. However, humanization of every amphipathic mouse residues is unnecessary because with minimal judicious amino acid substitutions, an AAR response can be minimized without jeopardizing the immunoreactivity, hence making it ideal for use in human therapeutics.

Key words: Antibody humanization, cell-based assay, immunogenicity, *Macaca fascicularis*, site-directed overlapping-PCR mutagenesis.

INTRODUCTION

The use of monoclonal antibodies (mAbs) generated from hybridoma technology for diagnosis and treatment of human cancers has been the subject of intense research for many years (Zafir-Lavie et al., 2007). Unfortunately, since mouse splenocytes are used, the resulting mouse mAbs are likely to induce an anti-antibody response (AAR), known as human anti-mouse antibody (HAMA) response when administered in humans (Hwang and Foote, 2005). Although the use of chimeric mAbs may reduce the AAR, because chimeric mAbs still contain approximately one third of antibody of mouse origin, it could still induce a significant AAR, known as human anti-chimeric antibody response (HACA). On the other hand, humanized mAbs which are commonly developed by complementary determining regions (CDR)-grafting method may have negligible AAR, but are often non-functional due to the loss of antigen-binding function. This

*Corresponding author. E-mail: sanjaydharshan@yahoo.co.uk. Tel: +60123959827. Fax: +6067997598.

is because some non-CDR mouse residues in the framework are still required for the effective binding of humanized mAbs and injudicious substitution of critical residues that maintain the CDR conformation required for specific binding to antigen may cause loss of binding ability (Mateo et al., 2000). Consequently, tedious and laborious back mutations are required to restore their functionality. Therefore, in the process of humanization, it would be desirable to substitute mouse amino acid residues in the variable region as judiciously as possible. A high degree of homology in the mouse and human framework will ensure that the CDR will have the greatest chance of retaining the binding properties. However, there will still be a certain degree of amino acid mismatch in these frameworks between human and mouse antibodies, which may give rise to AAR. Thus careful consideration of strategic substitution of these mismatched residues is needed for the humanized mAb to maintain binding affinity and at the same time induces minimize AAR when being administered in human.

In this communication, we describe the generation of humanized mAb against the C2 antigen, which is specifically expressed in colorectal carcinoma cells, from mouse mAbs. Here, only limited residues in the mouse framework region having the highest homology to the corresponding human framework region were considered for humanization. Humanized anti-C2 mAb (H2C2 mAb) was developed using logical approach method and was compared to our previous version of humanized anti-C2 mAb (H1C2 mAb), developed using deimmunization method (Roque-Navarro et al., 2003, Dharshanan et al., 2011a). The purity and functionality of both humanized mAbs were characterized *in vitro* using SDS-PAGE and cell-based assays, respectively, while the immunogenicity was characterized *in vivo* using *Macaca fascicularis* monkeys.

MATERIALS AND METHODS

Development of humanized mAbs

Construction of expression vectors with variable region of mouse antibody

The mouse VH and VL were constructed using a method described (Roque-Navarro et al., 2003). Briefly, total RNA was extracted from mouse hybridoma secreting anti-C2 antibody and was used to synthesize the cDNA of VH and VL. Both cDNAs were amplified using PCR and were ligated to pCR2.1-TOPO cloning vectors using TOPO-TA cloning kit (cat. no.: K4500-01, Life Technologies, USA). The resulting vectors were named mouse-pVH for VH and mouse-pVL for VL.

Humanization of mouse anti-C2 mAb using logical approach method

To determine the amino acids of VH and VL of mouse anti-C2 that differ with its homologous human amino acids, 100 most homologous human sequences for each VH and VL were obtained from the IgBLAST software (http://www.ncbi.nlm.nih.gov/igblast/). From the mouse-human amino acids comparison, the mouse framework with the highest homology to its corresponding human framework was determined. The mismatched mouse-human residues (if present) were considered candidates for substitution only if the corresponding human amino acid was present in 90% of the total human variable regions analyzed. Then the mismatched mouse-human residues in that framework were substituted with the residue occurring with the highest frequency in corresponding human framework.

Construction of expression vectors with variable region of human antibody

The substitution was executed by overlapping-PCR mutagenesis using primers shown in Table 1. Primers prefixed H and L was used for VH and VL, respectively. For both VH and VL, primers HF0-1 and LF0-2 were used as forward primers, while primers HR0-1 and LR0-2 were used as reverse primers. Primers numbered 1 and 2 were mutagenic primers containing nucleotide substitution (letters underlined and bolded) in its DNA sequence to introduce the specific amino acid conversion at the desired residues. All primers were synthesized by First BASE Laboratories (Selangor, Malaysia).

Humanization of residue 10 of mouse VH and residue 68 of mouse VL: Each of the site-specific mutagenesis was done by three sets of PCRs outlined below:

(i) PCR-1; the mixture contained 48.0 μl of master-mix components and 1.0 μl each of primers HF0/LF0 and HR1/LF1.
(ii) PCR-2 contained the exact components as PCR-1 except that primers HF1/LF1 and HR0/LF0 were used instead. The master-mix was made up of 10.0 μl of reaction buffer (10×), 2.0 μl of dNTPs (10 mM), 2.0 μl of recombinant *Taq* DNA polymerase, 3.0 μl of MgCl, 73.0 μl of sterile water and 6.0 μl of mouse-pVH/mouse-pVL vectors. The two PCR products were then purified before being merged using overlapping-PCR (PCR-3).
(iii) PCR-3 (overlapping-PCR); the mixture was similar to the master-mix of PCR-1 and 2 with the following exception, 76.0 μl of sterile water, 1.0 μl of recombinant *Taq* DNA polymerase, 1.0 μl of each primer HF0/LF0 and HR0/LR0 were used and the mouse-pVH/mouse-pVL vectors were substituted with 3.0 μl of each purified PCR-1 and PCR-2 products for a final volume of 100 μl. Vectors with the desired sequences were determined and named humanized-pVH1 (VH) and humanized-pVL1 (VL).

Humanization of residue 81 of mouse VL: The mutations of these residues were performed as described earlier with the following differences:

(i) Humanized-pVL1 (in both PCR-1 and PCR-2) was used instead of mouse-pVL vectors.
(ii) Primers LF1 and LR1 were replaced with LF2 and LR2, respectively. Vectors with the desired sequences were determined and named humanized-pVL2.

All PCR reactions were executed using the following steps: (1) 94°C for 3 min; (2) 94°C for 40 s; (3) 55°C for 30 s; (4) 72°C for 90 s; (5) repeat step 2 to 4 for 30 cycles; (6) 72°C for 10 min, and finally cooling to 4°C. The products of PCR-3 were purified, ligated

Table 1. List of primers and their DNA sequences. Primers with underlined and bold nucleotide are mutagenesis primers designed to introduce mutations at specific mouse residues to its homologous human residues

Primer name	Primer sequence
HF0	5'-GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT-3'
HR0	5'-GGGGCTAGCTGCAGAGACAGTGACCAGAGT-3'
HF1	5'-GAGTCAGGACCTG**G**CCTGGTGAAACCT-3'
HR1	5'-AGGTTTCACCAGG**C**CAGGTCCTGACTC-3'
LF0	5'-GGGGATATCCACCATGAGGTCCCCTGCTCAGCTC-3'
LR0	5'-AGCGTCGACTTACGTTTTATTTCCAGCTTGGTCCC-3'
LF1	5'-CCTGACAGATTCA**G**TGGCAGTGGATCA-3'
LR1	5'-TGATCCACTGCCA**C**TGAATCTGTCAGG-3'
LF2	5'-GATTTCGCACTGAAAATCAG**C**AGAGTG-3'
LR2	5'-CACTCT**G**CTGATTTTCAGTGCGAAATC-3'

to a cloning vector and transformed into competent bacterial cells. Plasmids were isolated from 24 bacterial clones and were sent for DNA sequence determination at First BASE Laboratories. The plasmids with the desired humanized VH and VL DNA sequences were then sub-cloned into expression vectors pAH4602, which contains the human IgG1 constant regions (for VH), and pAG4622, which contains the human kappa constant region (for VL) (Morrison et al., 1984). The recombinant vectors were named humanized-pAH4602 for humanized VH and humanized-pAG4622 for humanized VL.

Expression, production and purification of humanized antibodies

For the expression of H2C2 mAb, plasmids humanized-pAH4602 and humanized-pAG4622 were used. Both plasmids were co-transfected into NS0 cells and the high producer clones were selected using the ClonePix FL system. The best clone in terms of stability and productivity for each mAb, namely H2C2 (logical approach method), H1C2 (developed previously using deimmunization method), chimeric anti-C2 (QC2) and mouse anti-C2 (MC2) mAb was chosen and adapted to serum-free growth media (Dharshanan et al., 2011b). For small-scale production of mAbs, triple flasks were used and the secreted mAbs were purified by Protein A affinity chromatography using Äkta Prime plus.

Characterization of mAbs

SDS-PAGE

All purified mAbs were analyzed by SDS-PAGE under reducing and non-reducing conditions according to Laemmli (1970). The SDS-PAGE gel consisted of a 10% (v/v) separating gel topped by a 5% (v/v) stacking gel. Full range Rainbow marker (GE Healthcare, USA) was used as a reference for the estimation of the molecular weight of the protein bands.

In vitro binding studies

Immunofluorescence cell-based assay: Since it is critical to evaluate the functionality of hum-C2 mAbs after humanization, a cell-based ELISA was performed using a method described with modifications (Hong et al., 2004). The SW1116 colorectal cancer cell line (cat. no.: CCL-23, ATCC, USA) which expresses the C2 antigen on its cell surface was used. The cell line was grown at 37°C using Leibovitz's L-15 medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Biochrom, Germany). For the preparation of fixed cell-based ELISA, 96 well plates were cultured with 200 µl of 10^4 SW1116 cells/well and incubated at 37°C for 72 h. Plates were then washed three times with 200 µl of washing buffer (sterile water containing 0.09% sodium chloride and 0.05% tween-20) to remove previous media. The cells were then fixed using 200 µl of fixation solution and incubated at room temperature for 5 min. The fixation solution is a mixture of acetone and methanol at equal volumes. Wells were washed before they were blocked by the addition of 200 µl of blocking buffer (pH 7.4 phosphate buffer (1 M) containing 3% bovine serum albumin (BSA) and 0.1% tween-20) into each well and incubated at 37°C for 90 min. Wells were then washed 3 times with washing buffer. Next, 100 µl of 200 ng/ml purified H1C2 and H2C2 mAbs were added in triplicate to designated wells. For positive controls, wells were reacted with 100 µl of 200 ng/ml of mouse (MC2) and chimeric (QC2) anti-C2 mAbs. For negative controls, 100 µl of 200 ng/ml immunopure human IgG (cat. no.: 31154, Thermo Fisher Scientific, USA) and immunopure mouse IgG (cat. no.: 31202, Thermo Fisher Scientific, USA) were added. All samples were diluted using pH 7.4 phosphate buffer (1 M) prior to use. Wells were incubated at 37°C for 90 min and washed thrice with washing buffer, and binding of primary antibody was assessed using appropriate FITC-conjugated secondary antibodies. Anti-human secondary antibody was added to wells reacted with chimeric, humanized and human antibodies while anti-mouse secondary antibody was added to wells reacted with mouse antibodies. The secondary antibodies used were diluted to a ratio of 1:64 in phosphate buffer before use. Then, 100 µl of diluted secondary antibodies conjugated to FITC: anti-human detection agent-FITC (cat. no.: K8200, Molecular Devices, USA) or anti-mouse detection agent-FITC (cat. no.: K8220, Molecular Devices, USA) were added. The mixtures were incubated at 37°C for 60 min and unbound secondary antibodies were removed by washing with washing buffer before 50 µl of sterile water were added. The cell binding was visualized under a fluorescence microscope (Nikon, USA).

Competitive cell-based ELISA: To evaluate the affinity of the humanized mAb to C2 antigen, a competitive cell-based ELISA was performed. The fixation of SW1116 cells, blocking and washing of

the plates were similarly performed as described in immunofluorescence cell-based assay. Next, 50 µl of 0.1, 1.0 and 10.0 µg/ml of purified H1C2, H2C2 and QC2 mAbs were each mixed with 50 µl of 1.0 µg/ml of purified MC2 mAb. The mixtures were then added in triplicate to designated wells. For negative control, immunopureHuman IgG was used. The plates were incubated at 37°C for 90 min. Wells were washed 3 times with washing buffer and 100 µl of diluted secondary antibody: anti-mouse IgG conjugated to peroxidase enzyme (cat. no. A3673, Sigma-Aldrich) were added to each well. The secondary antibody used is specific to the γ-chain of mouse mAbs and was diluted to a ratio of 1:2500 in phosphate buffer before use. The mixtures were again incubated at 37°C for 60 min. Unbound secondary antibodies were removed by washing, and 100 µl ABTS substrate solution (cat. no. 11112422001 and 11112597001, Roche) were added. The mixture was incubated at room temperature in the absence of light for 30 min. Finally, the enzyme-substrate reaction was stopped by adding 50 µl of 0.5 M of sulfuric acid and absorbance was measured at 405 nm using a Synergy HT multi-mode microplate reader (BioTek, Winooski, Vermont, USA).

In vivo immunogenicity studies

To predict the immunogenicity of H1C2 and H2C2 mAbs in humans, non-human primates, macaque monkeys (*M. fascicularis)* were used. The use of monkeys for this study was approved by the Animal Care and Use Committee (ACUC), Laboratory Animal Science Centre, Faculty of Medicine, University of Malaya and granted ACUC ethics number: FIS/14/07/2010/SD (R). All procedures were performed on anesthetized *M. fascicularis* under the supervision of a veterinarian.

Immunization and blood collection of *M. fascicularis*: Five captive-born monkeys between 2 to 4 years old were obtained from Laboratory Animal Science Centre, University of Malaya. Each monkey was randomly chosen to be immunized with either with H1C2, H2C2, QC2, MC2 mAbs or placebo. For the first immunization, 0.2 mg mAb emulsified in 1 ml of complete Freund's adjuvant (CFA) (Sigma-Aldrich, USA) were injected into each monkey. For subsequent booster immunizations, CFA was replaced with incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, USA). Placebo consisted of adjuvants emulsified in the absence of mAb. Antibodies or placebo were injected intradermically at 5 different spots with approximately 0.2 ml per spot on day 0, 14, 28 and 42. Prior to each immunization, monkeys were anaesthetized with Zoletil (Virbac, USA) at a dose of 8 mg/kg through intramuscular injection. Then, the mass of each monkey was measured, and blood was collected on day 0, 14, 28, 42 and 56 from the cephalic vein using needle and syringe. Blood was immediately transferred to EDTA-treated tubes, inverted several times and centrifuged at 2000 rpm for 5 min at 4°C. The plasma samples were then aliquoted into small volumes into PCR tubes and stored at -20°C.

Measurement of monkeys' anti-antibody response: The AAR responses were determined by the measurement of blood IgM and IgG levels employing ELISA methods. Briefly, purified MC2 mAb (1 µg/ml) or purified QC2 mAb (1 µg/ml) were used as capture antibodies, anti-monkey IgM (cat. no.: 617-103-007, Rockland Immunochemicals, USA) and anti-monkey IgG (cat. no.: A2054, Sigma-Aldrich, USA) were used as the secondary antibodies to detect IgM and IgG levels, respectively. Both secondary antibodies are conjugated to peroxidase and were diluted to a ratio of 1:2500 in phosphate buffer before use. Monkey plasma samples were diluted to a ratio of 1:2500 and 1:5000 in phosphate buffer.

RESULTS

Development of humanized monoclonal antibodies

In the development of humanized mAb from mouse mAb, our principle was to judiciously and minimally substitute the amino acids on the variable region of mouse mAb in order to maximize probability of retention of reactivity and to minimize the incidence of AAR. In order to develop the humanized anti-C2 mAb, the VH and VL of mouse mAb were first aligned and compared to that of human in order to determine the frameworks that had the highest degree of homology. From the comparison, it was found that frameworks 1 and 3 for VH and VL had the highest degree of homology (75.9 and 87.8%, respectively) (Table 2a) and thus were chosen to be the targets for humanization. Due to the high degree of homology between mouse and human residues, it was felt that humanization on this framework would be least likely to affect the functionality of the humanized antibodies.

Using logical approach method to further minimize AAR, within each framework, the mismatched residues was humanized only if the corresponding human amino acid was present in at least 90% of all the homologous human variable regions analyzed. In framework 1 of VH, four mismatched mouse-human residues were present (Table 2b), but only aspartic acid at residue 10 was targeted for substitution to the corresponding human amino acid, glycine, which is the most abundant (91.2%) on the human framework. Coincidently, this residue was also humanized to glycine in the deimmunization method of H1C2 mAb. The other three mouse residues (16-Q, 17-S and 25-T) were not humanized because the corresponding human amino acids were present at less than 90% frequency (Table 2b). Similarly, three mismatched mouse-human residues were found in the framework 3 of VL (Figure 2c) but only two of the mismatched residues, 68 (threonine) and 81 (arginine) in mouse were substituted with the corresponding most abundant amino acid in the human framework, that is serine (100%) and serine (90.3%), respectively. Coincidently, residue 68-T was also humanized to serine in the deimmunization method of H1C2 mAb. Residue 88-L was not humanized because corresponding human amino acids were present with less than 90% frequency. It must be noted that other mismatched residues which are located in the CDR and Vernier zone, and therefore contribute to the antigen binding function of mAbs are not substituted (Roque-Navarro et al., 2003). The use of site-directed overlapping-PCR mutagenesis (Figure 1) has made it possible to develop H2C2 mAb with the amino acid substitutions at the desired residues. However, a large number DNA samples had to be sequenced because out of the 24 samples, only 12.5% to 25% of the purified DNA samples had the desired sequences. This may due to the high error rate of recombinant *Taq* DNA polymerase.

Table 2. Comparison of percentage homology between mouse and human framework regions (a) and percentage of mismatched mouse-human residues on VH (b) and VL (c) of anti-C2 monoclonal antibody obtained using IgBLAST. The mismatched mouse-human residues were humanized with the corresponding residues that are present in at least 90% of all the human residues analyzed (*).

(a) Frameworks	Homology (%)	
	VH	VL
1	75.9	82.0
2	71.0	80.0
3	67.7	87.8

(b) Framework 3-VL Mismatched residue	Mouse	Human (%)
*68	T	S (100.0)
*81	R	S (90.3) G (5.8) C (1.9) R (1.0) N (1.0)
88	L	F (64.1) V (24.3) E (5.8) A (2.9) I (1.9) T (1.0)

(c) Framework 1-VH Mismatched residue	Mouse	Human (%)
*10	D	G (91.2) T (6.8) V (1.0) I (1.0)
16	Q	E (44.7) Q (29.1) R (14.6) D (6.8) G (4.8)
17	S	T (85.4) S (14.6)
25	T	S (86.4) Y (12.6) T (1.0)

Characterization of mAbs

SDS-PAGE

From the SDS-PAGE analysis, bands of approximately 150 kDa were stained for all mAbs under non-reducing conditions (Figure 2a). Under reducing conditions (Figure 2b), two bands of approximately 55 and 25 kDa, corresponding to VH and VL, respectively, were identified for all mAbs. No other additional bands were detected

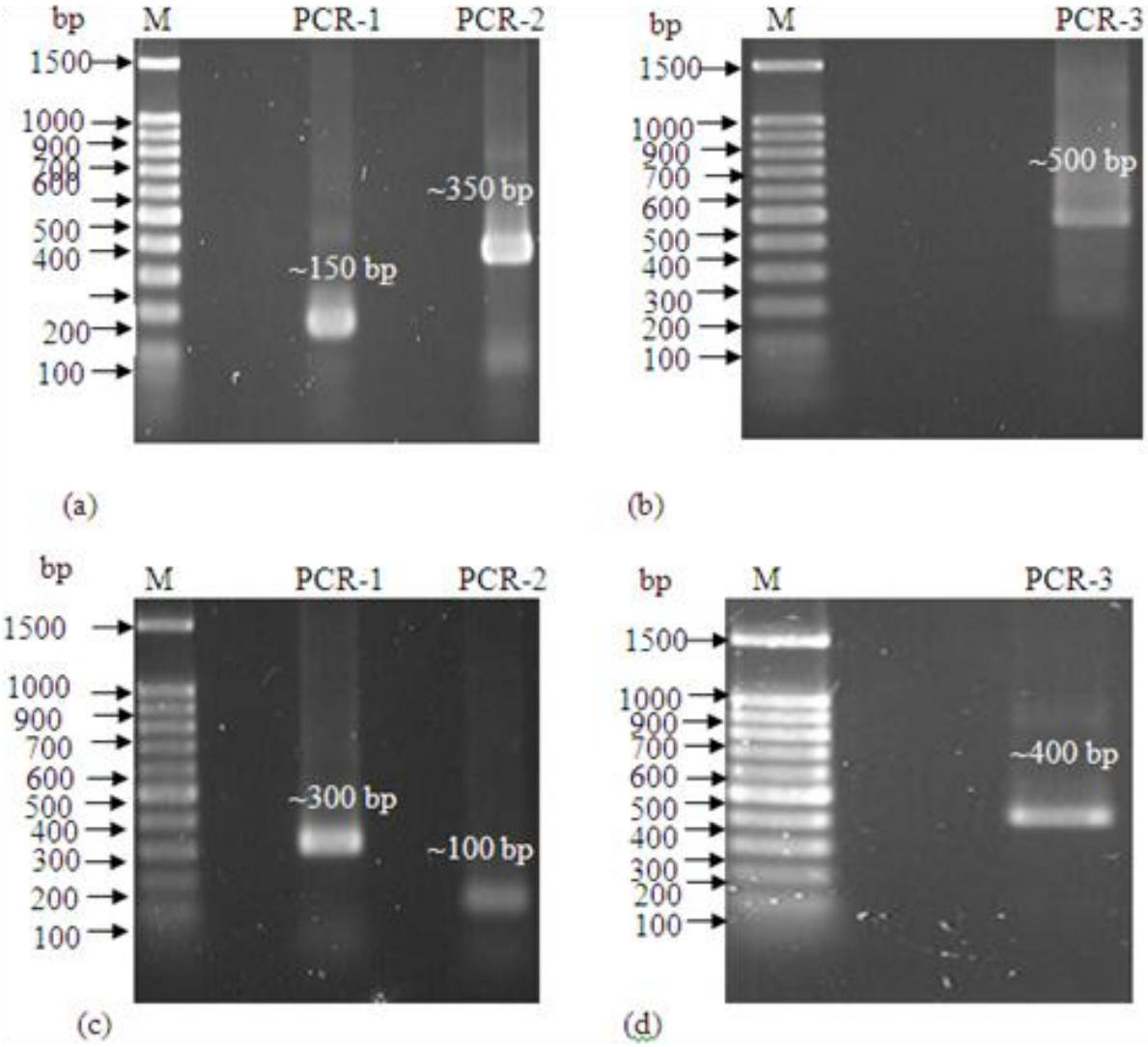

Figure 1. Agarose gel electrophoresis profiles of humanization of residue 10 of mouse VH (a and b) and 68 of VL (c and d). PCR-1 and PCR-2 are mutagenesis-PCR which introduces the specific amino acid humanization. PCR-3 is an overlapping-PCR which overlaps both PCR-1 and PCR-2 products. The products of PCR-1 and PCR-2 of VH (a) were approximately 150 and 350 bp, respectively, while that for VL (c) were approximately 300 and 100 bp, respectively. The products of PCR-3 of VH (b) and VL (d) were 500 and 400 bp, respectively. 100 bp DNA ladder (M) was used as reference.

under both non-reducing and reducing conditions, indicating that all the purified mAbs were not contaminated with significant quantities of endogenous orexogenous proteins. Since mAbs were produced in serum-free environment, all the purified mAbs were free from bovine polyclonal IgG antibody contamination.

In vitro binding studies

Immunofluorescence assay: A critical issue concerning humanized antibodies is the possible loss of binding ability after modifications are made to reduce their immunogenicity. Therefore, the functionality of the humanized antibodies is usually evaluated using conventional ELISA which requires antigens in purified form. Given the lack of commercially-available purified form of C2 antigen, a cell-based ELISA was used instead (Vazquez et al., 1995; Solano et al., 2003; Ramos-Suzarte et al., 2007). This assay uses SW1116 cell which is a colorectal adenocarcinoma cell-line expressing C2 antigen on its surface as the capture antigen in what is otherwise a conventional antigen-based ELISA. Since SW1116 cells are adherent cells, live SW1116 cells were used initially to avoid potential alteration of the epitope on the C2 antigen caused by drying or fixation (Hong et al., 2004). Although cell-binding was observed, the variation in binding of the triplicate wells with live cells was

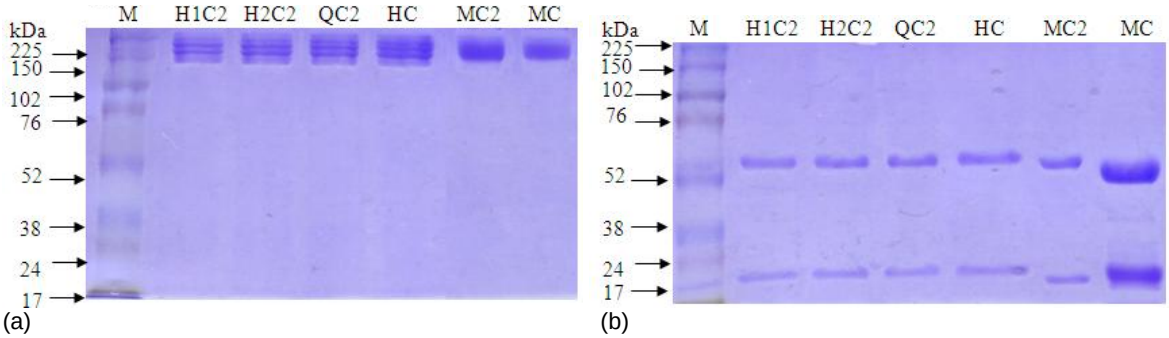

Figure 2. *In vitro* characterization of purified mAbs using SDS-PAGE under non-reducing (a) and reducing conditions (b). From the SDS-PAGE analysis, bands of approximately 150 kDa were stained for all mAbs under non-reducing conditions. Under reducing conditions, two bands of approximately 55 and 25 kDa, corresponding to the VH and VL, were identified for all mAbs. No additional bands were detected under both conditions.

significantly higher compared to the situation where fixed SW1116 cells were used. Microscopic examination revealed that significant loss of non-fixed live SW1116 cells occurred during washing. In contrast, the use of fixation solution might have anchored cells firmly in the wells, but still preserved the structure of C2 antigen and therefore decreased the experimental variation for consistent and reproducible cell-binding ELISA results (Yang et al., 2003).

From the cell-based IFA with H1C2, H2C2, QC2 and MC2 mAbs (Figure 3), high fluorescence images were obtained compared to wells where unspecific human and mouse antibodies were used. Intense fluorescence was seen in wells incubated with H1C1 and H2C2 mAbs, as with QC2 and MC2 mAbs. This shows that both H1C2 and H2C2 mAbs were still able to bind to C2 antigen expressed on SW1116 cells. This is to be expected because no humanization was done on mouse residues in the CDR or Vernier zone which are both important for antigen recognition (Mateo et al., 2000).

Competitive cell-based ELISA: Figure 4 compares the immunoreactivity of H1C2, H2C2 and QC2 mAbs to C2 antigen expressed on SW1116 colorectal carcinoma. Competitive ELISA showed that MC2 mAb was inhibited in a dose-dependent manner by increasing concentrations of chimeric and humanized anti-C2 mAbs. Half displacement concentrations of H1C2, H2C2 and QC2 mAbs were 1.6, 1.7 and 0.85 μg/ml, respectively, (Figure 4). Thus, the mutations introduced in the variable region of humanized anti-C2 mAbs provoked approximately 50% reduction in binding affinity for both H1C2 and H2C2 mAbs.

In vivo immunogenicity studies

The next critical issue was whether the humanized mAbs developed by logical approach method would have a decreased immunogenicity when they were administered in humans. To address this issue, *M. fascicularis* were used because of the similarity of monkeys' immune system to that of humans', thus providing a reliable indicator of potential human AAR. Although *M. fascicularis* are only listed in appendix II (currently not threatened with extinction) by Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), in Malaysia, however, it is classified as a protected animal, under schedule two of the Protection of Wild Life Act 76, 1972. Due to the stringent laws and regulations in Malaysia, only five captive-born *M. fascicularis* were permitted to be used for this study. For immunization, the intradermal route was chosen because of the high population of matured dendritic cells in the dermis layer of the skin (Cui et al., 2003). During the study, no evident changes in behavior, food consumption and body mass were observed in all *M. fascicularis*. Compared to *M. fascicularis* immunized with humanized and chimeric anti-C2 mAbs, the *M. fascicularis* immunized with MC2 mAb had the highest monkey IgG and IgM AAR irrespective whether MC2 mAb (Figure 5) or QC2 mAb (Figure 6) was used as capture antibody. Decreased but significant AAR responses were obtained when *M. fascicularis* were immunized with QC2 and H2C2 mAbs. However, it can be seen that when H1C2 mAb was used as immunogen, the monkey's AAR was similar to that of those immunized with placebo (Figures 5 and 6).

DISCUSSION

In general, H2C2 mAb was still able to bind to C2 antigen expressed on SW1116 cells, which shows that the humanized mAb developed using logical approach method was still functional. In terms of affinity, a reduction of ~50% was observed for H2C2 mAb (logical approach method) and also in H1C2 mAb (deimmunization method). Nevertheless, while both H1C2 and H2C2 mAbs had reduced immunogenicity

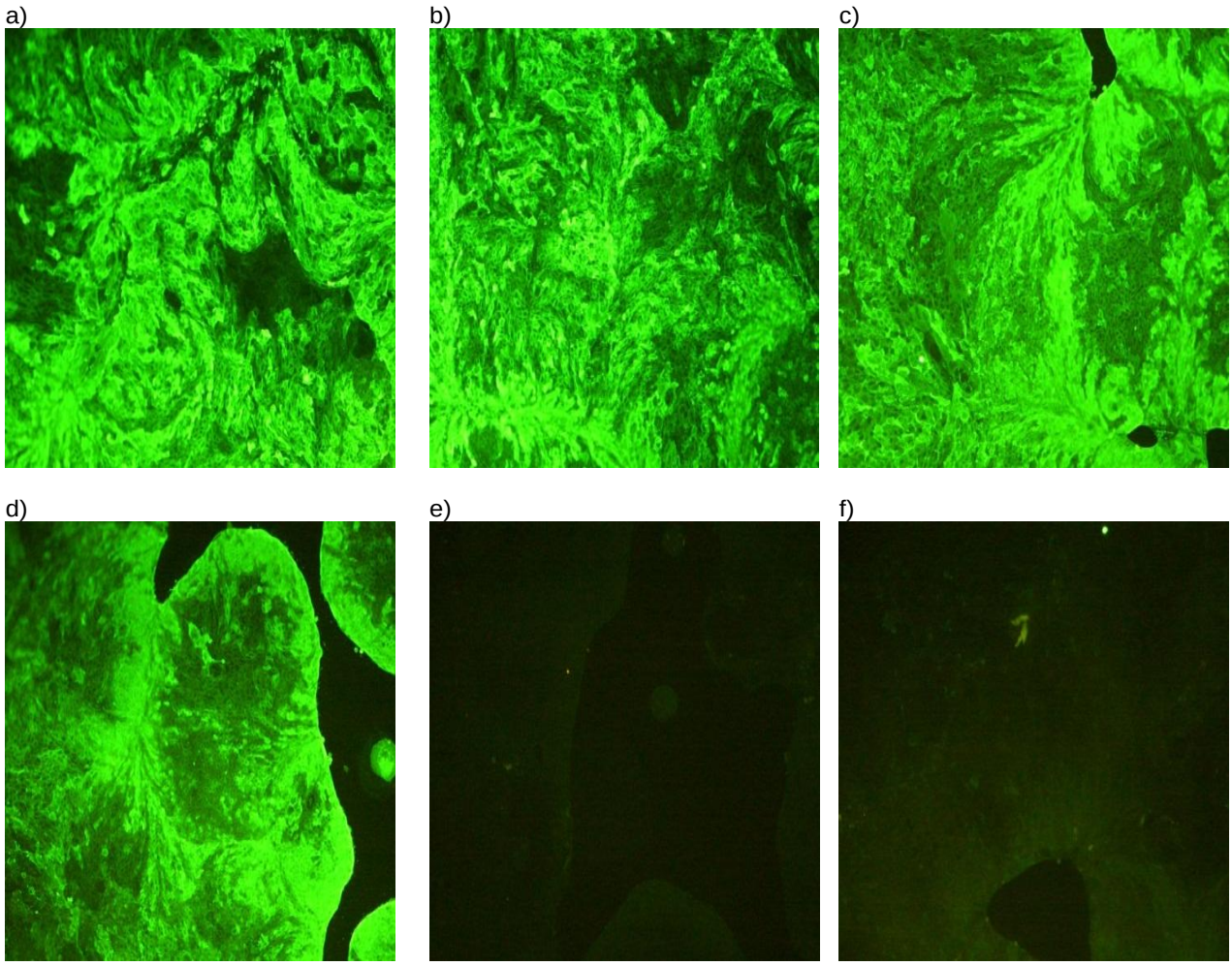

Figure 3. *In vitro* characterization of purified mAbs by cell-based IFA using colorectal carcinoma expressing C2 antigen. From the cell-based studies, cell-binding were observed as indicated by the high fluorescence in images obtained when H1C2 (a), H2C2 (b), QC2 (c) and MC2 (d) mAbs were used, compared to the lack of fluorescence in images using unspecific human (e) and mouse (f) control antibodies. This demonstrates that even after humanization both H1C2 and H2C2 mAbs developed using deimmunization and logical approach methods, respectively, were still functional.

compared to MC2 mAb, however only H1C2 mAb had its immunogenicity virtually eliminated. On the other hand, the immunogenicity of H2C2 mAb was still similar to that of QC2 mAb. The reduced immunogenicity of H1C2 mAb compared to H2C2 mAb may be due to the fact that H1C2 mAb have less amphipathic regions compared to that of H2C2 mAb. In VH of H1C2 mAb, the humanized residues 10 and 17 are located in the first amphipathic region, whereas the remaining humanized residues 44 and 45 are located in the second amphipathic region (Roque-Navarro et al., 2003). Similarly, in VL of H1C2 mAb, the humanized residue 15 is located in the first amphipathic region and residue 50 is located in the second amphipathic region. Therefore, both chains of H1C2 mAb had only 1 remaining amphipathic region; VH (third amphipathic region) and VL (third amphipathic region).

On the other hand, the H2C2 mAb developed using logical approach method had one humanized residue 10 in VH which is in the first amphipathic region and 2 humanized residues 68 and 81 which are in the second amphipathic region in VL. Thus, H2C2 mAb have two remaining amphipathic regions in each VH (second and third amphipathic regions) and in VL (first and third amphipathic regions). The additional amphipathic regions may have caused the immunogenicity of H2C2 mAb to be higher that of H1C2 mAb. The deimmunization method combines veneering (based on Padlan's approach) (Mateo et al., 1997) to effectively humanize surface residues (thus removing B-cell epitopes) with the identification and removal of potential helper T-cell epitopes from engineered antibodies. Helper T-cell epitopes are short peptide sequences within proteins that bind to MHC class II molecules. The peptide-MHC

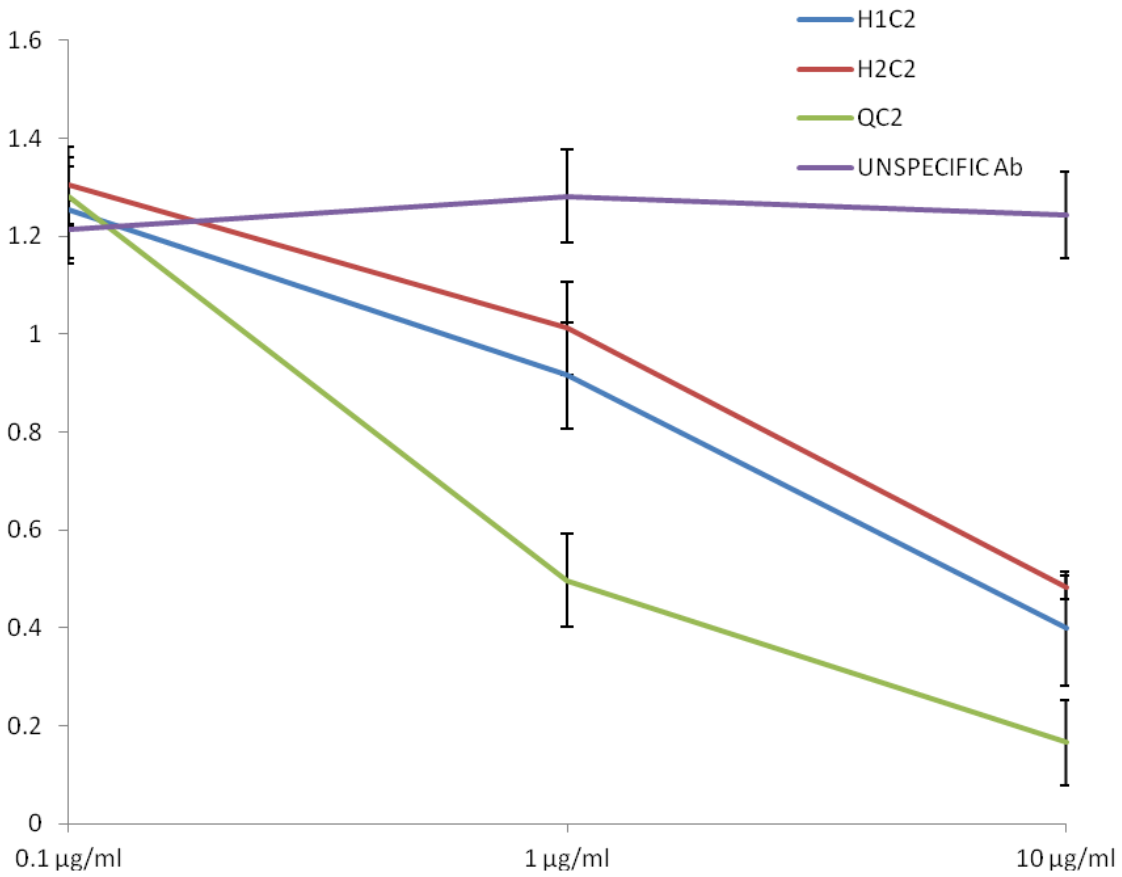

Figure 4. *In vitro* reactivity of purified antibody reactivity measured by competitive cell-based ELISA. Displacement of MC2 mAb binding to C2 antigen, a membrane glycoprotein expressed in SW1116 human colorectal cell line-coated plates, by H1C2 (blue line), H2C2 (red line), and QC2 (green line) mAbs. An unspecific human mAb (purple line) was included as negative control.

class II complexes are recognized by T-cells, and trigger the activation and differentiation of helper T-cells, thus stimulating a cellular immune response. Helper T-cells initiate and maintain immunogenicity by interacting with B-cells, resulting in the production of antibodies that bind specifically to the administered antibody. In deimmunization method, helper T-cell epitopes are identified within the primary sequence of the antibody using prediction software and these sequences are altered by amino acid substitution to avoid recognition by T-cells. Thus, the nature of the protein surface is important for its recognition by the monkey or human immune system because the protein internalization and processing by antigen-presenting cells and the presentation of processed peptides to T-helper cells in the context of class II major histocompatibilty complex molecules are also essential events for the development of an immune response against administered mAbs (Mateo et al., 2000). Therefore, the removal of most linear epitopes that may have been presented to T-cells had considerably reduced the immunogenicity of H1C2 mAb. In contrast, the presence of additional potential T-cell epitopes of H2C2 mAb even after humanization may have contributed to its higher immunogenicity.

Hence the deimmunization method is a superior method for the development of functional humanized mAbs with reduced immunogenicity. The fact that, deimmunization method had also been applied previously on ior-egf/r3, a mouse mAb which blocks the epidermal growth factor receptor (EFGR) and the resulting humanized antibody retained its antigen-binding affinity and was less immunogenic in monkeys than either their mouse or chimeric predecessors, further proves the superiority of deimmunization method compared to logical approach method (Mateo et al., 2000).

Conclusion

In conclusion, although the small number of monkeys permitted for this study prelude statistical analysis of the data, it is noteworthy that *M. fascicularis* immunized with humanized anti-C2 (H2C2) mAb had reduced immunogenicity compared to *M. fascicularis* immunized

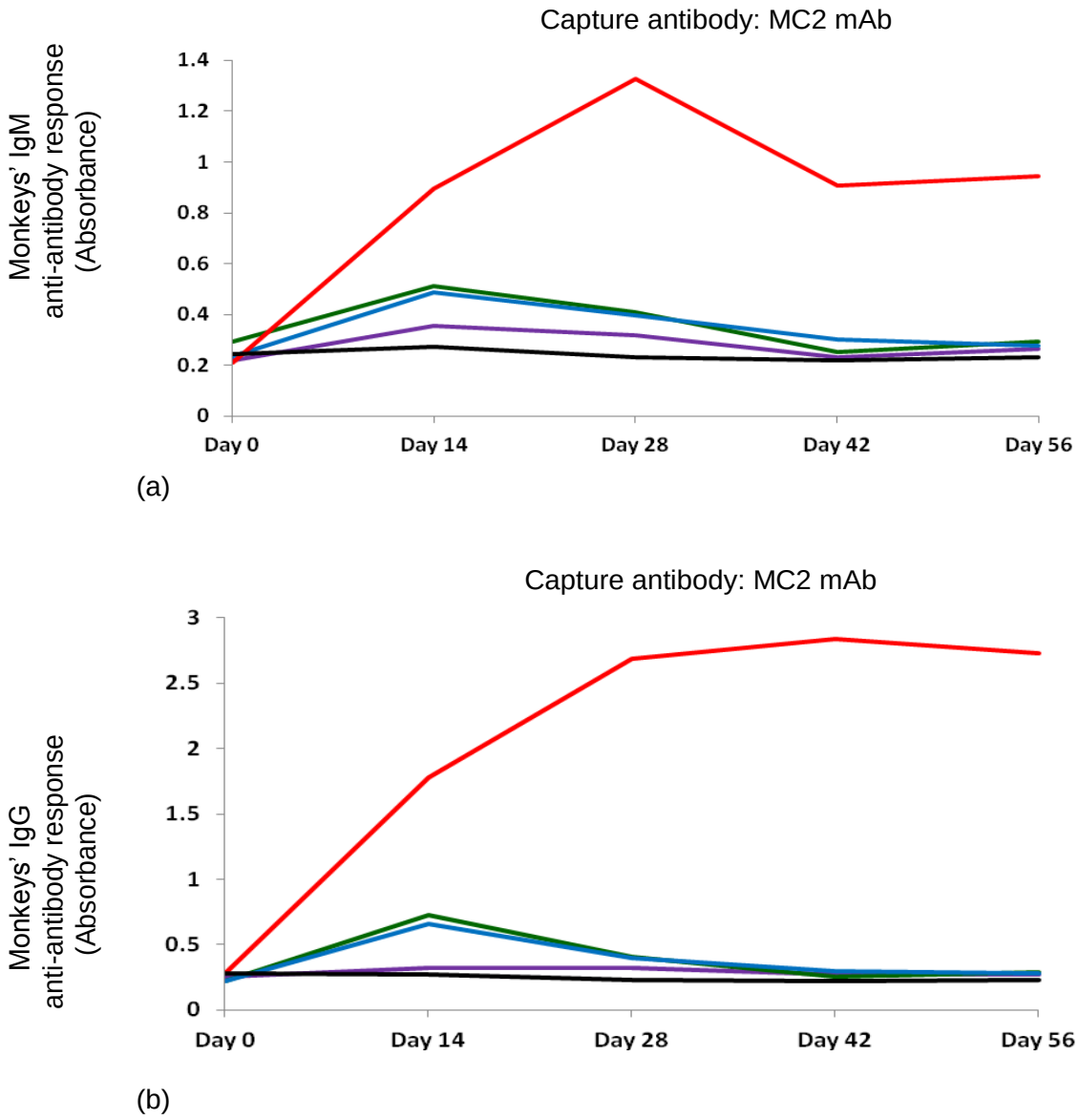

Figure 5. Monkeys' anti-antibody responses to mouse-, chimeric- and humanized anti-C2 antibodies using MC2 mAb as capture antibody. These graphs show the AAR of monkeys to MC2 (red), QC2 (blue), H1C2 (purple), and H2C2 (green) mAbs as measured by titers of IgM (a) and IgG (b). MC2 mAbs were used as capture antibody. For negative control, *M. fascicularis* was immunized with placebo (black).

with mouse anti-C2 mAb. While both humanized anti-C2 mAbs; H1C2 and H2C2 mAbs developed by deimmunization and logical approach method, respectively, are functional and have 50% reduction in binding affinity, the reduced immunogenicity using logical approach method however is not guaranteed as indicated by H2C2 mAb. Therefore, for the development of functional humanized mAbs with reduced AAR, it is recommended that amphipathic mouse residues be targeted for humanization, while those located in the CDR or Vernier zone be left alone. Therefore, it is concluded that the deimmunization method should be used to humanize mouse monoclonal antibodies. The retention of functionality and induction of minimal AAR make humanized H1C2 mAbs ideal for use in human therapeutics.

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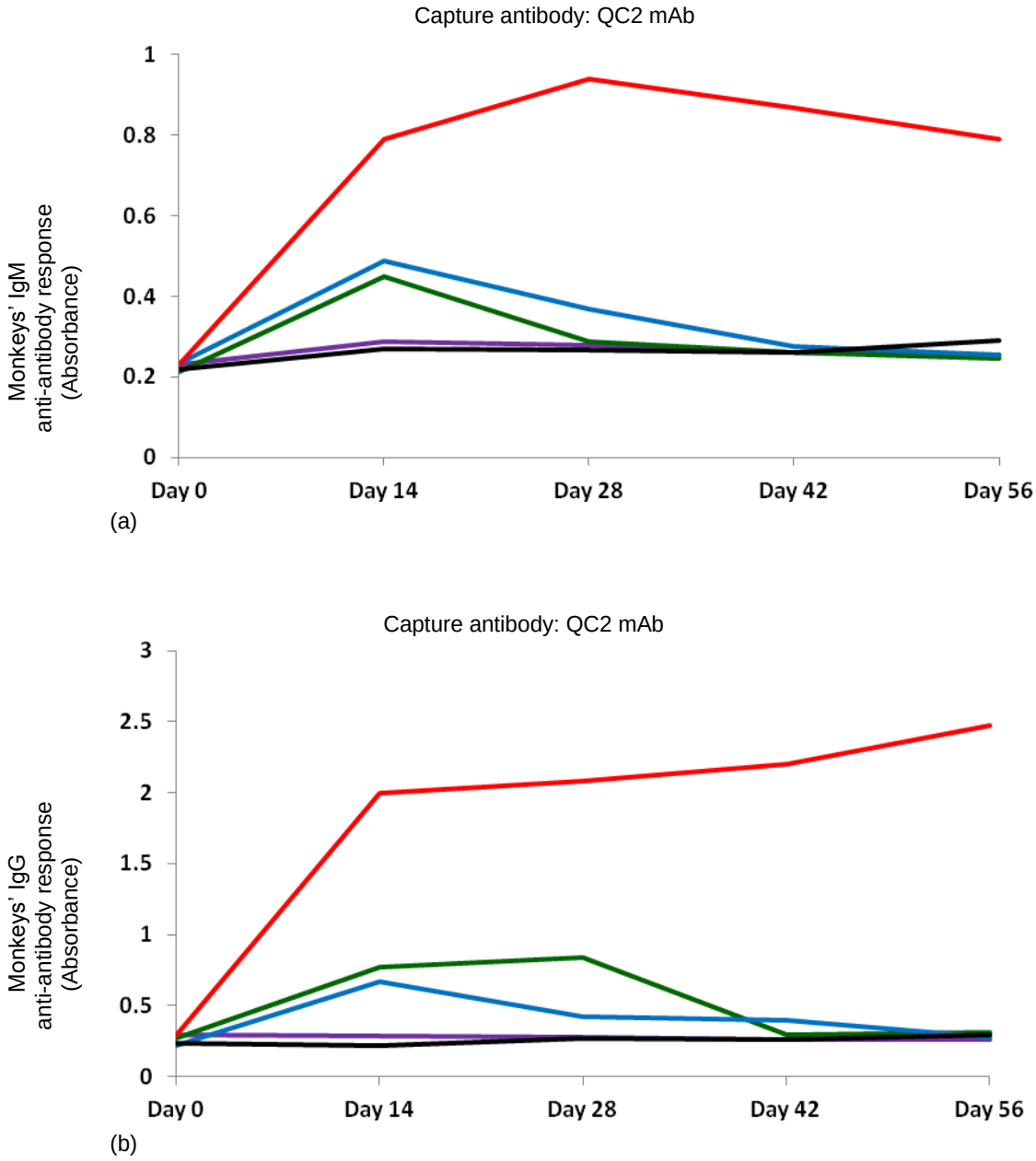

Figure 6. Monkeys' anti-antibody responses to mouse-, chimeric- and humanized anti-C2 antibodies using QC2 mAb as capture antibody. These graphs show the AAR of monkeys to MC2 (red), QC2 (blue), H1C2 (purple), and H2C2 (green) mAbs as measured by titers of IgM (a) and IgG (b). QC2 mAbs were used as capture antibody. For negative control, *M. fascicularis* was immunized with placebo (black).

Abbreviations: AAR, Anti-antibody response; **CDR,** complementary determining regions; **H1C2,** humanized anti-C2 mAb developed using deimmunization method; **H2C2,** humanized anti-C2 mAb developed using logical approach method; **IFA,** immunofluorescence assay; ***M. fascicularis*,** *Macacca fascicularis*; **mAb,** monoclonal antibody; **MC2,** mouse anti-C2 mAb; **QC2,** chimeric anti-] C2 mAb; **VH,** variable region of the heavy chain; **VL,** variable region of the light chain.

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